IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty's Docket No. 101141-12

APPLICANT : German A. Valcarce

FILED : Concurrently Herewith

FOR : Cholesterol Desaturases from Ciliates, Methods and Uses

PRELIMINARY AMENDMENT

Hon. Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the application as follows:

IN THE SPECIFICATION

Page 1, after line 1, please insert --This application is a continuation-in-part application of United States Serial Number 09/835,804 filed April 16, 2001; which was a continuation-in-part application of United States Serial Number 09/641,609 filed August 17, 2000, which claimed the benefit of United States provisional application serial numbers 60/153,754 filed September 13 1999, 60/153,741 filed September 13, 1999, 60/172,844 filed December 20, 1999, and 60/177,252 filed January 20, 2000.--

IN THE CLAIMS

Please amend the claims as follows. A marked-up copy of the amended claims is enclosed.

4. (amended) A process for manufacturing Δ7 dehydrochlesterol (provitamin D3) and Δ 7,22 bis dehydrocholesterol comprising:

(a) mixing a cell free extract from Ciliate phylum microorganism, wherein said cell free extract contains cholesterol desaturase activities selected from the group comprising Δ-7 and Δ-22 cholesterol desaturases activities that catalyze desaturation of cholesterol with a cholesterol substrate;

(b) incubating the mixture for a period of time enough to produce Δ7 dehydrocholesterol and Δ7,22 bis dehydrocholesterol;

(c) recovering said Δ7 dehydrocholesterol and Δ7,22 bis dehydrocholesterol by solvent extraction and chromatographic purification.

11. (amended) A process for preparing a substantial pure Δ7 cholesterol desaturase enzyme from Ciliata phylum microorganism wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ7 dehydrocholesterol by introducing a double bound at the position seven in the cholesterol molecule, the process comprising the steps of:

(a) culturing a microorganism in a suitable medium, wherein said microorganism is capable of producing Δ7 cholesterol desaturases;

(b) disintegrating the culture and extracting the same with buffer solution containing, if necessary, non ionic surfactant or stabilizer as glycerol;

(c) subjecting the extract to a chromatography purification under suitable conditions; and

(d) eluting and recovering said Δ7 cholesterol desaturases.

14. (amended) A process for preparing a substantial pure Δ22 cholesterol desaturase enzyme from Ciliata phylum microorganism, wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ22 dehydrocholesterol by introducing a double bound at the position twenty-two in the cholesterol molecule the process comprising the steps of:

(a) culturing a microorganism in a suitable medium, wherein said microorganism is capable of producing Δ22 cholesterol desaturases;

(b) disintegrating the culture and extracting the same with buffer solution containing, if necessary, non ionic surfactant or stabilizer as glycerol;

(c) subjecting the extract to a chromatography purification on a suitable chromatography conditions; and

(d) eluting and recovering said Δ22 cholesterol desaturases.

REMARKS

The above amendments were made to place the application into proper United States Patent Format.

Respectfully Submitted,

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Amended Claims - Marked-up Copy

1. A cell free extract from Ciliate phylum microorganism, wherein said cell free extract contains cholesterol desaturase activities selected from the group comprising Δ-7 and Δ-22 cholesterol desaturases activities that catalyze desaturation of cholesterol.

2. A cell free extract of Claim 1, wherein said cell free extract is selected from the group consisting of cell free homogenate, microsomal fraction and desaturase-enriched fraction, or a combination thereof, all from Ciliata phylum microorganism.

3. A cell free extract of Claim 1, wherein the ciliate is selected from the group consisting of *Paremecium*, *Tetrahymena* and *Colpidium*.

4. (amended) A process for manufacturing Δ7 dehydrochlesterol (provitamin D3) and Δ 7,22 bis dehydrocholesterol comprising:

(a) mixing a cell free extract ~~of claim 1~~ from Ciliate phylum microorganism, wherein said cell free extract contains cholesterol desaturase activities selected from the group comprising Δ-7 and Δ-22 cholesterol desaturases activities that catalyze desaturation of cholesterol with a cholesterol substrate;

(b) incubating the mixture for a period of time enough to produce Δ7 dehydrocholesterol and Δ7,22 bis dehydrocholesterol;

(c) recovering said Δ7 dehydrocholesterol and Δ7,22 bis dehydrocholesterol by solvent extraction and chromatographic purification.

5. A substantial pure Δ7 cholesterol desaturase enzyme from Ciliata phylum microorganism, wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ7 dehydrocholesterol by introducing a double bound at the position seven in the cholesterol molecule.

6. A substantial pure Δ7 cholesterol desaturase enzyme of Claim 5, wherein the ciliate is selected from the group consisting of *Paremecium, Tetrahymena* and *Colpidium*.

7. A substantial pure Δ7 cholesterol desaturase enzyme according to claim 5, the enzyme

(a) having a molecular weight of approximately 60 kDa by gel chromatography;

(b) having an optimum pH range for enzymatic activity between 6.5-8.5;

(c) having an optimum temperature range for enzymatic activity of 28°C to 35°C;

(d) being unaffected by metal ions such as Ca^{+2}, Mn^{+2} and Mg^{+2} ,EDTA concentrations and 2-mercaptoethanol;

(e) being inactivated after 1 minute at 100°C;

(f) being storage at -20°C by at least 6 months.

8. A substantial pure Δ22 cholesterol desaturase enzyme from Ciliata phylum microorganism, wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ22 dehydrocholesterol by introducing a double bound at the position twenty-two in the cholesterol molecule.

9. A substantial pure Δ22 cholesterol desaturase enzyme of Claim 8, wherein the ciliate is selected from the group consisting of *Paremecium, Tetrahymena* and *Colpidium*.

10. A substantial pure Δ22 cholesterol desaturase enzyme according to claim 8, the enzyme

(a) having a molecular weight of approximately 60 kDa by gel chromatography;

(b) having an optimum pH range for enzymatic activity between 5.5-8.5;

(c) having an optimum temperature range for enzymatic activity of 28°C to 35°C;

(d) being unaffected by metal ions such as Ca^{+2}, Mn^{+2} and Mg^{+2} and EDTA concentrations;

(e) being inactivated after 1 minute at 100°C;

(f) being storage at -20°C by at least 6 months.

11. (amended) A process for preparing a substantial pure Δ7 cholesterol desaturase enzyme from Ciliata phylum microorganism ~~according to claim 5~~ wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ7 dehydrocholesterol by introducing a double bound at the position seven in the cholesterol molecule, the process comprising the steps of:

(a) culturing a microorganism in a suitable medium, wherein said microorganism is capable of producing Δ7 cholesterol desaturases;

(b) disintegrating the culture and extracting the same with buffer solution containing, if necessary, non ionic surfactant or stabilizer as glycerol;

(c) subjecting the extract to a chromatography purification under suitable conditions; and

(d) eluting and recovering said Δ7 cholesterol desaturases.

12. The process according the claim 11, wherein the step of culturing is carried out in a medium containing 1% proteose peptone, 0.1% yeast extract, 0.5% glucose, 0.01% Sequestrene and 0,5mg% of 22 dehydrocholesterol.

13. The process according the claim 11, wherein the chromatography purification is selected from a group comprising

size exclusion chromatography, anion exchange chromatography, cation exchange chromatography and combinations thereof.

14. (amended) A process for preparing a substantial pure Δ22 cholesterol desaturase enzyme from Ciliata phylum microorganism ~~according to claim 8~~, wherein said enzyme is capable of catalyzing the conversion of a cholesterol substrate in Δ22 dehydrocholesterol by introducing a double bound at the position twenty-two in the cholesterol molecule the process comprising the steps of:

(a) culturing a microorganism in a suitable medium, wherein said microorganism is capable of producing Δ22 cholesterol desaturases;

(b) disintegrating the culture and extracting the same with buffer solution containing, if necessary, non ionic surfactant or stabilizer as glycerol;

(c) subjecting the extract to a chromatography purification on a suitable chromatography conditions; and

(d) eluting and recovering said Δ22 cholesterol desaturases.

15. The process according the claim 14, wherein the step of culturing is carried out in a medium containing 1% proteose peptone, 0.1% yeast extract, 0.5% glucose, 0.01% Sequestrene and 1.0 mg% of cholesterol.

16. The process according the claim 14, wherein the chromatography purification is selected from a group comprising size exclusion chromatography, anion exchange chromatography, cation exchange chromatography and combinations thereof.

~~17. The use of substantial pure Δ7 cholesterol desaturase enzyme from Ciliata phylum microorganism of claim 5 for producing Δ7 dehydrocholesterol (provitamin D3) employing cholesterol as substrate.~~

~~18. The use according the claim 17, wherein the cholesterol substrate es seleccionado del grupo comprendido por colesterol puro, cholesterol containing products and cholesterol enriched fractions.~~

~~19. The use according the claim 17, wherein the ciliate is selected from the group consisting of *Paremecium*, *Tetrahymena* and *Colpidium*.~~

~~20. The use of pure Δ7 cholesterol desaturase and substantial pure Δ22 cholesterol desaturase enzymes from Ciliata phylum microorganism of claims 5 and 8 for producing Δ7,22 bis dehydrocholesterol employing cholesterol as substrate.~~

~~21. The use according the claim 20, wherein the cholesterol substrate es seleccionado del grupo comprendido por~~

~~colesterol puro, cholesterol containing products and cholesterol enriched fractions.~~

~~22. The use according the claim 20, wherein the ciliate is selected from the group consisting of *Paremecium, Tetrahymena* and *Colpidium.*~~